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Covalent modification of the catalytic sites of the H^+-ATPase from chloroplasts with 2-nitreno-ADP. Modification of the catalytic site 1 (tight) and catalytic sites 1 and 2 together impairs both uni-site and multi-site catalysis of ATP synthesis and ATP hydrolysis

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Abstract

After isolation and purification, the H^+-ATPase from chloroplasts, CF_0F_1, contains one endogenous ADP at a catalytic site, and two endogenous ATP at non-catalytic sites. Incubation with 2-azido-[α-^{32}P]ADP leads to tight binding of azido-nucleotides. Free nucleotides were removed by three consecutive passages through centrifugation columns, and upon UV-irradiation most of the label was covalently bound. The labelled enzyme was digested by trypsin, the peptides were separated by ion exchange chromatography into nitreno-AMP, nitreno-ADP and nitreno-ATP labelled peptides, and these were then separated by reversed phase chromatography. Amino acid sequence analysis was used to identify the type of the nucleotide binding site. After incubation with 2-azido-[α-^{32}P]ADP, the covalently bound label was found exclusively at β-Tyr-362. Incubation conditions with 2-azido-[α-^{32}P]ADP were varied, and conditions were found which allow selective binding of the label to different catalytic sites, designated as 1, 2 and 3 in order of decreasing affinity for ADP, and either catalytic site 1 or catalytic sites 1 and 2 together were labelled. For measurements of the degree of inhibition by covalent modification, CF_0F_1 was reconstituted into phosphatidylcholine liposomes, and the membranes were energised by an acid-base transition in the presence of a K^+/valinomycin diffusion potential. The rate of ATP synthesis was 50–80 s^{-1}, and the rate of ATP hydrolysis was 15 s^{-1} measured under multi-site conditions. Covalent modification of either catalytic site 1 or catalytic sites 1 and 2 together inhibited ATP synthesis and ATP hydrolysis equally, the degree of inhibition being proportional to the degree of modification. Extrapolation to complete inhibition indicates that derivatisation of catalytic site 1 leads to complete inhibition when 1 mol 2-nitreno-ADP is bound per mol CF_0F_1. Derivatisation of catalytic sites 1 and 2 together extrapolates to complete inhibition when 2 mol 2-nitreno-ADP are bound per CF_0F_1. The rate of ATP synthesis and the rate of ATP hydrolysis were measured as a function of the substrate concentration from multi-site to uni-site conditions with derivatised CF_0F_1 and with non-derivatised CF_0F_1. ATP synthesis and ATP hydrolysis under uni-site and under multi-site condition were inhibited by covalent modification of either catalytic site 1 or catalytic sites 1 and 2 together. The results indicate that

Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Tricine, *N*-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]glycine; CF_0F_1, proton translocating H^+-ATPase from chloroplasts; HPLC, high pressure liquid chromatography; AXP, AMP or ADP or ATP

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derivatisation of site 1 inhibits activation of the enzyme and that cooperative interactions occur at least between the catalytic sites 2 and 3.

Keywords: H^+ATPase; CF_0F_1; Nucleotide binding; Uni-site catalysis; 2-Azido-nucleotide

1. Introduction

H^+-ATPases ('ATP synthase', F-type ATPase, F_0F_1) catalyse ATP synthesis and ATP hydrolysis in bacteria, chloroplasts and mitochondria. They use the free enthalpy difference ('Gibbs free energy') derived from the transmembrane proton transport to synthesise the β-γ-phosphate-bond in ATP from ADP and phosphate. All F-type H^+-ATPases have a similar structure; they consist of a membrane-inserted F_0-part involved in proton translocation and a hydrophilic F_1-part containing the nucleotide and phosphate binding sites [1–5]. Photolabile as well as chemically reactive nucleotide analogues have been used to identify the nucleotide binding sites within the protein. 2-Azido-AD(T)P has been frequently used, since it has the same 'anti' conformation as the adenine nucleotides, and its photolabile group is small, so that binding to the enzyme is not disturbed [6–9]. Thus, the analogue binds to F-ATPases with similar characteristics as AD(T)P, and it is a substrate for both ATP synthesis and ATP hydrolysis with almost the same K_m values as those of the corresponding adenine nucleotides [10–12].

After photolysis of bound 2-azido-AD(T)P, the resulting 2-nitreno-AD(T)P is exclusively bound to the β-subunit of CF_0F_1 [13]. Detailed analysis of the labelled amino acids under different conditions gave the following result: when binding and covalent labelling of catalytic sites was analysed, the nitreno-[α-^{32}P]ADP appeared to be bound at Tyr-362 of the β-subunit. Binding to non-catalytic sites resulted in the labelling of Tyr-385 of the β-subunit [14,15]. This surprising result was understood after the elucidation of the structure of MF_1 at 0.28 nm resolution [16]: the catalytic sites are located on the β-subunits and β-Tyr-362 (CF_1-numbering) is located near the azido-group of 2-azido-ADP. The non-catalytic sites are located on the α-subunits, but a stretch of the neighbouring β-subunit forms a part of the binding site and this stretch contains β-Tyr-385 which is located near the azido group of 2-azido-ATP bound at the α-subunit.

Whereas agreement has been achieved on the number of nucleotide binding sites, the interaction between the different sites is still an open question. In rotational catalysis all three catalytic sites participate sequentially in catalysis. At each moment, the three sites have a different structure, but during catalysis, they undergo conformational changes, adopting the open, tight and loose conformation in sequential order [17,18]. However, there are also reports that only two β-sites are directly involved in multi-site catalysis, the third site containing a tightly bound nucleotide in MF_1 [19] or turning over very slowly in CF_0F_1 [20,21]. Covalent derivatisation of catalytic sites and investigation of its effect on catalysis has been frequently used for clarifying this question. When all catalytic sites operate sequentially, covalent modification of one site is expected to block enzyme activity completely. However, earlier studies on the correlation between derivatisation of the F_1-parts of F-type ATPases from different sources gave no clear-cut result (for review, see [22,23]). The main problems were the following: first, not only one type of nucleotide binding sites was derivatised. Second, covalent modification of one catalytic site of CF_1 with 2-azido-ATP does not cause a complete loss of enzymatic activity, and additional ^{18}O measurements indicate formation of catalytic sites with altered properties [24]. Third, covalent derivatisation of one site might lead to different effects (full inhibition, partial inhibition, no inhibition) on the rate catalysed by the non-derivatised sites. These observations impede the interpretation of the correlation between activity and enzyme modification. For a mechanistic interpretation, there are two additional problems. First, most of the work with covalent labelling of nucleotide binding sites was not carried out with the holoenzyme, but with the experimentally more amenable F_1-part. F_1-catalysed ATP hydrolysis, however, only gives information on a partial reaction of the enzyme, and it is important to know, how catalytic

sites interact, when ATP synthesis and ATP hydrolysis are coupled with proton transport. Second, a special problem arises with the chloroplast enzyme, since CF_1 is usually obtained in an inactive latent form and requires one or another special (mis-) treatment in order to induce ATP hydrolysis activity. Such treatments might generate a heterogeneity of the enzymes and/or a heterogeneity of catalytic pathways.

Purified CF_0F_1 can be reconstituted into liposomes, and, after energisation of the membrane by $\Delta pH/\Delta\phi$, the rate of ATP synthesis is almost as high as in thylakoid membranes [27]. In this system, it is possible to correlate the effect of covalent modification with the inhibition of the complete reaction cycle including H^+-translocation. Based on earlier work on nucleotide binding to CF_1 [25,26], we investigated the correlation between covalent derivatisation and ATP synthesis with reconstituted CF_0F_1 under conditions, when only one type of nucleotide binding site was derivatised.

The three sites on the β-subunits will be called sites 1, 2 and 3 in order of decreasing affinity to ADP. Correspondingly, the three α-sites are called sites 4, 5 and 6 in order of decreasing affinity to ATP. Recently, we have shown that upon specific covalent modification of either catalytic site 2 (loose) or catalytic site 3 (open) multi-site catalysis is inhibited proportionally to the fraction of covalently modified enzyme molecules, while uni-site catalysis is not affected [28]. In the present study we have covalently modified site 1 (tight), and site 1 and site 2 together and investigated the effect on catalysis. The data indicate that in this case, both uni-site and multi-site catalysis are inhibited proportionally to the fraction of modified enzymes.

2. Materials and methods

The H^+-ATPase from chloroplasts was isolated and purified as described earlier [29], except that no nucleotides were present in the sucrose-density gradient centrifugation. The enzyme was obtained in a solution containing 300 g/l sucrose, 30 mM Tris-succinate, pH 6.5, 0.5 mM EDTA, 2 g/l Triton X-100 (Sigma) and 1 g/l asolectin (Fluka) with a protein concentration of about 2–5 g/l. It was rapidly frozen and stored in liquid nitrogen. Protein concentration was measured with the Bio-Rad protein assay using bovine serum albumin as a standard. A molecular mass of 550 kDa for the CF_0F_1 was assumed. After three consecutive passages through Sephadex G-50 centrifugation columns [30] equilibrated in buffer A (20 mM Na-succinate, 20 mM Na-Tricine, pH 8.0, 80 mM NaCl), the CF_0F_1 contains approximately 2 bound ATP and 1 bound ADP per enzyme.

The synthesis of 2-azido-[α-^{32}P]AD(T)P was performed as described in [31,32]. The specific activity after synthesis was 900–1000 dpm/pmol. The 2-azido-[α-^{32}P]ADP contained maximally 1% 2-azido-[α-^{32}P]-ATP. Incubation with 2-azido-[α-^{32}P]AD(T)P, photoaffinity labelling and identification of the modified binding site was carried out as described earlier [28,33]. The reconstitution of CF_0F_1 into liposomes was performed as described [35]. The lipid concentration of the proteoliposome suspension was 10 mM, the concentration of reconstituted CF_0F_1 was 80 nM for measurements under multi-site conditions, and 800 nM for measurements under uni- and bi-site conditions. After reconstitution, the enzyme was reduced by incubation with 50 mM dithiothreitol for 2 h at room temperature. Endogenous nucleotides were measured as described in [21]. The rates of ATP synthesis and ATP hydrolysis were measured after a $\Delta pH/\Delta\phi$ jump as described [28,34–36].

3. Results

3.1. Experimental protocol

After isolation and purification of CF_0F_1, free and loosely bound nucleotides were removed by three consecutive passages through centrifugation columns. After this treatment, the enzyme contains about one ADP and two ATP per CF_0F_1, the ADP being bound at a β-subunit and the two ATP molecules at α-subunits [28]. This enzyme was used for the following experiments: CF_0F_1 was incubated with 2 mM EDTA with different concentrations of 2-azido-[α-^{32}P]ADP for up to 5 h at room temperature. After incubation, free nucleotides were again removed by three consecutive centrifugation columns, and protein concentration and 2-azido-[α-^{32}P]nucleotides and endogenous nucleotides were

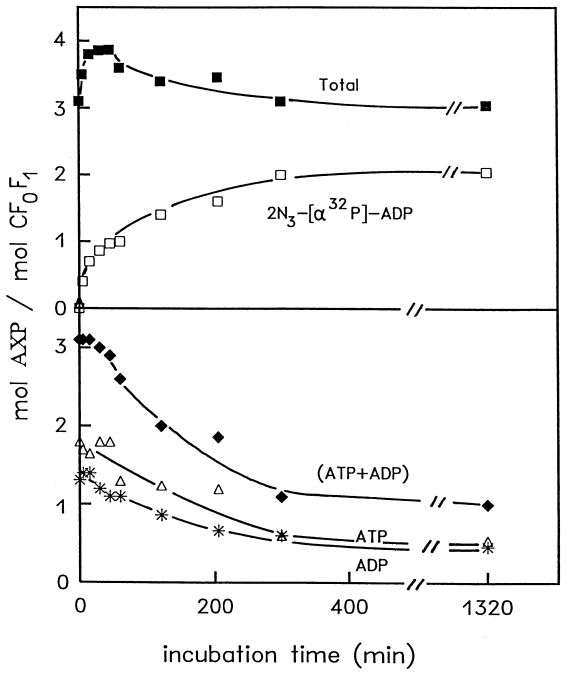

Fig. 1. Binding of 2-azido-[α-^{32}P]ADP to CF_0F_1. Isolated CF_0F_1 (5 μM) was incubated with 100 μM 2-azido-[α-^{32}P]ADP in the presence of 2 mM EDTA up to 22 h. After different incubation times, free nucleotides were removed by three consecutive passages through centrifugation columns, the enzyme was denatured and the nucleotide content was determined. Top panel: the binding of 2-azido-[α-^{32}P]ADP and total nucleotide content. Bottom panel: the endogenous nucleotide content. About one endogenous nucleotide and two 2-azido-[α-^{32}P]ADP were bound per CF_0F_1 after 5 h.

measured. Then, a part of the enzyme preparation with the bound 2-azido-[α-^{32}P]ADP was irradiated with UV-light for covalent modification and digested by trypsin. The labelled peptides were separated by HPLC and the amino acid sequence of the labelled peptides was determined. In parallel, the irradiated and, separately, the non-irradiated enzymes were reconstituted into liposomes. Again the endogenous nucleotide- and 2-azido-[α-^{32}P]ADP content were determined after reconstitution. Finally, the rate of ATP synthesis and ATP hydrolysis was measured as a function of the ADP or ATP concentration from uni-site to multi-site conditions. The same measurements were carried out with the non-irradiated part of CF_0F_1 which contained non-covalently bound 2-azido-[α-^{32}P]ADP. Additionally, the release of bound 2-azido-[α-^{32}P]ADP was measured after energisation in the presence as well as in the absence of a catalytic turnover.

As a control, isolated CF_0F_1 without incubation with 2-azido-[α-^{32}P]ADP was either irradiated with UV-light or not irradiated. Again, both enzymes were reconstituted separately into liposomes and the nucleotide content, ATP synthesis and ATP hydrolysis were measured. As a second control, isolated CF_0F_1 was incubated with ADP in the same way as with 2-azido-[α-^{32}P]ADP and then reconstituted into

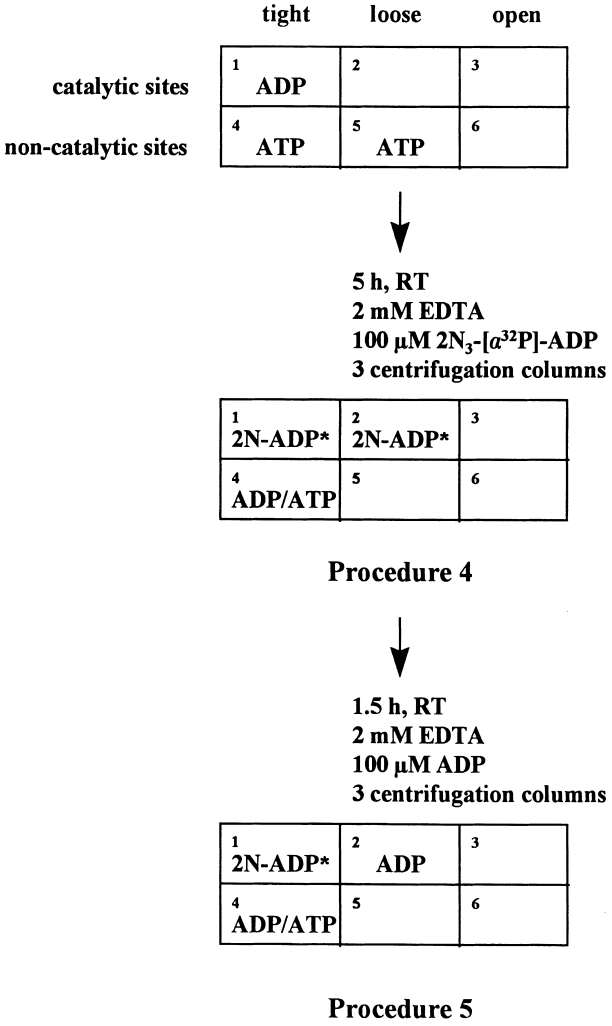

Fig. 2. Scheme of the procedures for specific labelling of catalytic sites 1 and 2. Procedure 4 leads to occupation of two sites, procedure 5 to occupation of only one site (site 1). For clarity, the resulting occupation of the binding sites is shown already in this figure, although the arguments for this assignment are given in Section 4.

liposomes. None of these treatments changes the results of the activity measurements.

3.2. Binding of 2-azido-[α-^{32}P]ADP

Fig. 1 shows the variation of the endogenous nucleotide content and the binding of 2-azido-[α-^{32}P]-ADP when CF_0F_1 is incubated with 100 μM 2-azido-[α-^{32}P]ADP and 2 mM EDTA. In the first 50 min, binding of one 2-azido-[α-^{32}P]ADP per CF_0F_1 is observed without a significant change of the endogenous ADP and ATP content. Then, one endogenous nucleotide, mainly ATP, is released, and the binding of a second 2-azido-[α-^{32}P]ADP per CF_0F_1 occurs which is complete after about 5 h. The binding of the second 2-azido-[α-^{32}P]ADP is accompanied with the release of one endogenous nucleotide (mainly ADP) so that, finally, the total nucleotide content is again three nucleotides per CF_0F_1. Further incubation up to 22 h does not change this distribution (see Fig. 1).

This treatment is called procedure 4 and leads to the occupation of two catalytic binding sites with 2-azido-[α-^{32}P]ADP. Fig. 2 shows schematically procedure 4 and the resulting occupation of the binding sites. Additionally, Fig. 2 shows the treatment which leads to occupation of catalytic site 1 (procedure 5). In order to clarify the presentation, we have already included the result for the occupation pattern of the binding sites, although the arguments for this assignment are given later.

The enzyme treated according to procedure 4 contained 1.8 bound 2-azido-[α-^{32}P]ADP and one residual endogenous nucleotide (see Table 1 and Fig. 2).

Table 1
Characterisation of isolated CF_0F_1 and of CF_0F_1 reconstituted into liposomes and analysis of covalently bound nucleotides after UV-irradiation

Incubation conditions	Isolated CF_0F_1				CF_0F_1 reconstituted into liposomes				Analysis of irradiated CF_0F_1		
	ATP_{bound}	ADP_{bound}	ADP_{free}	2-N_3-[α-^{32}P]-AXP	ATP_{bound}	ADP_{bound}	ADP_{free}	2-N_3-[α-^{32}P]-AXP	covalently bound 2-N_3-[α-^{32}P]-AXP	% AXP-labelled peptide	labelled Tyr
Incubation in buffer A, 30 min	1.75	1.30	0.015	0	0.70	1.45	0.45	0	–	–	–
Procedure 4: incubation in buffer A+ 2 mM EDTA+ 100 μM 2-N_3-[α-^{32}P]ADP, 5 h	0.40	0.70	0.05	1.8	0.15	0.40	0.40	1.7	1.4	−ADP >95%	Y-362
Procedure 5: as procedure 4, followed by incubation in buffer A+ 2 mM EDTA+ 100 μM ADP, 1.5 h	0.40	1.5	0.09	1.1	0.30	1.50	0.30	0.95	0.80	−ADP >95%	Y-362

Data are given in mol nucleotide per mol CF_0F_1. No free ATP was detected under any condition. The % of AXP-labelled peptides is given as percent of radioactivity of the sample applied on the ion exchange column. Incubations were carried out at room temperature. After each incubation, free nucleotides were removed by three consecutive passages of the CF_0F_1 solution through centrifugation columns. The data represent the average from three experiments (initial conditions average of 12 preparations). The standard deviation was less than 10% for all data shown.

This enzyme was then irradiated with UV-light for up to 60 s. After each irradiation time, the protein was denatured and the amount of covalently bound 2-nitreno-[α-^{32}P]AXP was measured (Table 2). The data indicate that maximally 75% of the bound 2-azido-[α-^{32}P]ADP can be covalently bound under these conditions. In order to measure the effect of covalent modification on ATP synthesis activity, the enzyme was reconstituted into liposomes after each irradiation time, and, after a $\Delta pH/\Delta\phi$ jump, the rate of ATP synthesis was measured with 100 μM ADP. It decreased from 88 to 14 s^{-1} after 60 s of irradiation (Table 2). In order to distinguish the effect of covalent modification of the binding sites from the potentially harmful effect of UV-irradiation, the enzyme was treated according to procedure 4, but the 2-azido-[α-^{32}P]ADP was substituted by ADP. This enzyme was irradiated in the same way, reconstituted into liposomes and the rate of ATP synthesis was measured. Up to an irradiation time of 30 s the rate remains constant and a small decrease is observed after 60 s irradiation (Table 2).

Fig. 3 shows the rate of ATP synthesis at different levels of covalently bound 2-nitreno-[α-^{32}P]ADP. Extrapolation of the linear part of the curve indicates complete inhibition, when about two nucleotides are covalently bound.

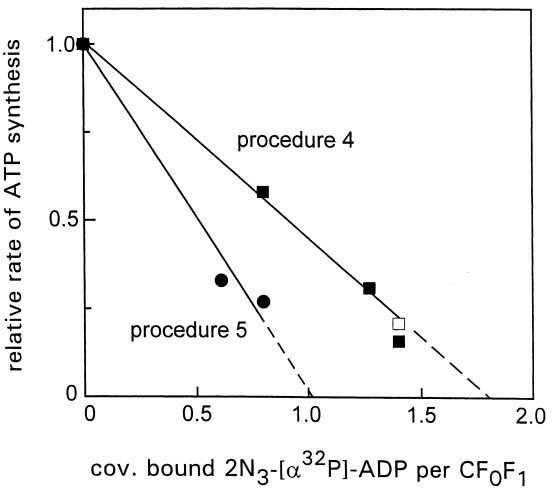

Fig. 3. Relative rate of ATP synthesis as a function of the amount of covalently bound 2-azido-[α-^{32}P]ADP. CF_0F_1 (5 μM) was incubated with 100 μM 2-azido-[α-^{32}P]ADP in presence of EDTA for 5 h. Free nucleotides were removed by three consecutive passages through centrifugation columns (procedure 4). The enzyme treated according to procedure 4 was then incubated with 100 μM ADP in the presence of EDTA for 1.5 h and free nucleotides were removed by three passages through centrifugation columns (procedure 5). Both enzymes were covalently labelled by UV-irradiation for different times. After reconstitution of the differently labelled enzymes into liposomes, the rate of ATP synthesis was measured, and the data are plotted as a function of covalently bound 2-nitreno-[α-^{32}P]ADP per CF_0F_1. The rate of ATP synthesis without covalent derivatisation were 88 s^{-1} for the enzyme treated with procedure 4 and 55 s^{-1} for the enzyme treated with procedure 5.

3.3. Localisation of the bound 2-nitreno-ADP

The enzyme treated according to procedure 4 was irradiated for 60 s with UV-light, then digested with

Table 2
Effect of UV-irradiation on covalent labelling of CF_0F_1 and on the rate of ATP synthesis

Irradiation time (s)	Rate of ATP synthesis (s^{-1}) Control	Total bound 2-N_3-[α-^{32}P]ADP	Covalently bound 2-N_3-[α-^{32}P]ADP	Rate of ATP synthesis (s^{-1})
Procedure 4				
0	83	1.8	0	88
15	88	1.9	0.79	51
30	85	1.8	1.27	27
60	67	1.8	1.4	14
Procedure 5				
0	52	0.9	0	55
15	52	0.9	0.5	25
30	55	1.0	0.8	15

The enzyme was incubated with 2-azido-[α-^{32}P]ADP according to procedure 4 or procedure 5. As a control, the enzyme was either treated according to procedure 4 or to procedure 5, however, ADP was used instead of 2-azido-[α-^{32}P]ADP. Data are given in mol nucleotide per mol CF_0F_1.

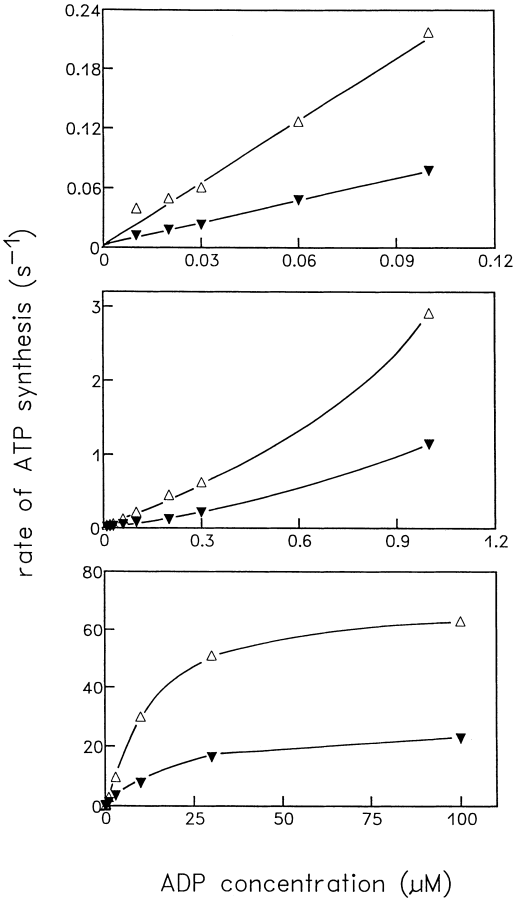

Fig. 4. ATP synthesis of CF_0F_1 with covalently bound and non-covalently bound 2-azido-[α-^{32}P]ADP at sites 1 and 2 as a function of the ADP concentration. CF_0F_1 was incubated as described under procedure 4. The CF_0F_1 contained 1.4 covalently bound 2-azido-[α-^{32}P]ADP (filled triangles) or 2 non-covalently bound 2-azido-[α-^{32}P]ADP (open triangles) after reconstitution into liposomes. The rate of ATP synthesis was measured after ΔpH/Δϕ jump. Top panel: data under uni-site conditions (up to 100 nM ADP). Bottom panel: data under multi-site conditions (up to 100 μM ADP). Center panel: the intermediate range.

trypsin, and the resulting peptides were separated by HPLC. Almost all radioactivity was present as nitreno-ADP-labelled peptides. Nitreno-AMP-labelled peptides represent less than 5% of the total radioactivity, taking into account that the radioactivity in the void volume appeared to be nitreno-AMP-labelled peptides. The elution profile of the HPLC separation on the reversed phase column indicated that more than 95% of the radioactivity represents peptides of the catalytic site and sequence analysis indicates that only β-Tyr-362 was derivatised (see Table 1). Obviously, both sites occupied with procedure 4 are catalytic sites [14,15].

3.4. *Effect of modification of two catalytic sites on the kinetics of the enzyme*

In order to characterise these occupied binding sites we investigated the effect of their covalent derivatisation on the catalytic properties of the enzyme. CF_0F_1 containing two 2-azido-[α-^{32}P]ADP (procedure 4) at catalytic sites was irradiated for 60 s to achieve maximal derivatisation (1.4 mol bound 2-nitreno-[α-^{32}P]ADP per mol CF_0F_1). The irradiated enzyme and, as a control the non-irradiated enzyme were reconstituted into liposomes. ATP synthesis was measured after a ΔpH/Δϕ jump with ADP concentrations ranging from 10 nM to 100 μM. The initial rates of ATP synthesis as a function of ADP concentration are depicted in Fig. 4. At low ADP concentrations (uni-site conditions, top panel), medium

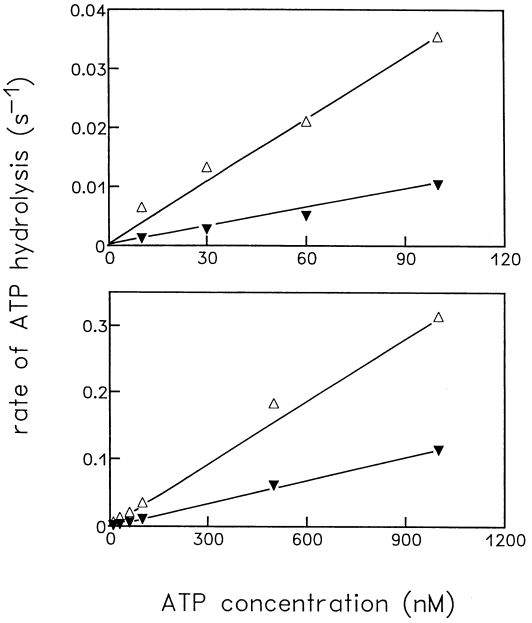

Fig. 5. ATP hydrolysis of CF_0F_1 with covalently bound and non-covalently bound 2-azido-[α-^{32}P]ADP at site 1 and site 2 as a function of the ATP concentration. CF_0F_1 was incubated according to procedure 4. CF_0F_1 contained after reconstitution into liposomes 2.0 non-covalently bound 2-azido-[α-^{32}P]ADP per CF_0F_1 (open triangles) or 1.4 covalently bound 2-azido-[α-^{32}P]ADP per CF_0F_1 (filled triangles). The rate of ATP hydrolysis was measured after activation of the enzyme by a ΔpH/Δϕ jump from uni-site (upper panel) to multi-site (lower panel) conditions.

ADP concentrations (centre panel) and high ADP concentrations (multi-site conditions, bottom panel) a strong inhibition of the rate of ATP synthesis by covalent derivatisation is observed. Approximately 70% of the two initially bound 2-azido-[α-^{32}P]ADP are covalently bound after irradiation and, in parallel, the rate of ATP synthesis decreased by 70% at all ADP concentrations.

In addition, the rate of ATP hydrolysis was measured with the irradiated and non-irradiated CF_0F_1. First, the reconstituted enzyme was activated by a $\Delta pH/\Delta\phi$ jump and then the activated enzyme was injected into a buffer containing an uncoupler, the luciferin/luciferase assay and different ATP concentrations [28]. Fig. 5 shows the initial rates of ATP hydrolysis at low (uni-site conditions, top panel) and medium concentrations (bottom panel). At high ATP concentrations (1 mM, multi-site conditions) the rate was measured with [α-^{32}P]ATP [36]. With the non-covalently labelled enzyme the rate was 17 s^{-1}, with the covalently derivatised enzyme 4 s^{-1}. At all ATP concentrations from uni-site to multi-site, the rate decreases by approximately 70% after covalent derivatisation. Correspondingly, 70% of the two initially bound 2-azido-[α-^{32}P]ADP are covalently bound after irradiation, i.e. at all concentrations the inhibition of the rate is proportional to the fraction of covalently derivatised enzyme. These data indicate that covalent labelling of catalytic sites 1 and 2 inhibits both ATP synthesis and ATP hydrolysis from uni-site to multi-site conditions to the same extent. This is surprising since selective covalent derivatisation of catalytic site 2 does not lead to inhibition of uni-site catalysis [28]. We have to conclude that inhibition of uni-site catalysis results from the covalent derivatisation of catalytic site 1. In order to distinguish between the effect of the two different sites, we have developed procedure 5 which allows selective labelling of catalytic site 1.

3.5. *Effect of covalent modification of the catalytic site 1 on the enzyme activity*

When isolated CF_0F_1 was treated according to procedure 4, the enzyme contains finally about two 2-azido-[α-^{32}P]ADP and one endogenous nucleotide per CF_0F_1. This enzyme was subjected to a second incubation in the presence of 2 mM EDTA and 100

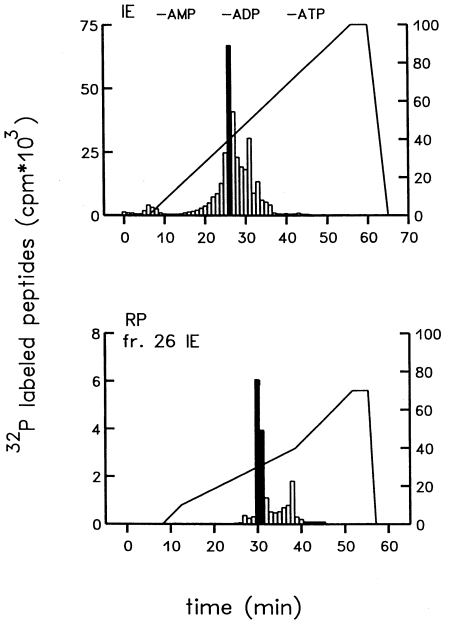

Fig. 6. HPLC elution profiles of photolabelled CF_0F_1 after trypsin treatment. CF_0F_1 (5 μM) was treated according to procedure 5 and 2-azido-[α-^{32}P]ADP was covalently bound to the enzyme by UV-irradiation for 30 s. The photolabelled CF_0F_1 was treated with trypsin and the resultant labelled peptides were separated by HPLC. The radioactivity of the collected fractions is represented by bars. The elution gradients in presence of eluent B are superimposed (right hand scale). Upper panel shows the separation of the labelled peptides with the ion exchange chromatography. Lower panel represents the result of the separation of nitreno-ADP derivatised peptides (fraction 26 from ion exchange chromatography) by a reversed phase chromatography. Amino acid sequence analysis was carried out with fraction 30. The nitreno-ADP was found on of the β-Tyr-362.

μM ADP for 1.5 h (see procedure 5, Fig. 2). After removal of free nucleotides, we obtained an enzyme containing 1.1 bound 2-azido-[α-^{32}P]ADP, 1.5 bound ADP and 0.4 bound ATP per CF_0F_1 (Table 1).

This enzyme was then irradiated with UV-light for up to 30 s and the covalently bound 2-nitreno-ADP was measured as described before. Table 2 shows the results. After 30 s of UV-irradiation, 0.78 mol 2-nitreno-[α-^{32}P]ADP per mol CF_0F_1 was covalently bound. After reconstitution of the covalently derivatised CF_0F_1 into liposomes, the rate of ATP synthesis was measured after a $\Delta pH/\Delta\phi$ jump. It decreased from 52 to 15 s^{-1} after 30 s of irradiation (see Table 2). The data are plotted in Fig. 3. Extrapolation of the linear part of the curve indicates complete inhi-

bition when one nucleotide is bound. As a control, the enzyme incubated with ADP only, was irradiated. After reconstitution, the rate of ATP synthesis was measured and no decrease of the rate was observed up to an irradiation time of 30 s.

3.6. Localisation of the bound 2-nitreno-ADP

CF_0F_1 was treated according to procedure 5 and irradiated with UV-light for 30 s. The protein was then digested with trypsin and the resulting peptides were separated by HPLC. Fig. 6 top shows the elution profile of an HPLC anion exchange column run. The bars represent the radioactivity of the collected fractions, the solid line indicates the concentration of eluent B. The expected regions for nitreno-AMP-, nitreno-ADP- and nitreno-ATP-containing peptides are indicated. No nitreno-ATP-labelled peptides were found, the nitreno-AMP-labelled peptides represent less than 5% of the total radioactivity, i.e. almost all radioactivity eluted as nitreno-ADP. Fraction 26 was subjected to a second HPLC run, using a reversed phase column, and the fraction eluting at 30 min (Fig. 6 bottom) was used for amino acid sequencing. The sequence was identified as: ^{360}GI-PAVDPLDSTST...^{378}, Tyr-362 being the missing derivatised amino acid. Obviously, under these conditions (procedure 5), only a catalytic site was labelled. Since the nucleotide at this catalytic site is most difficult to exchange, this site is catalytic site 1 (tight catalytic site).

In Table 1 the data of the analysis of the isolated and reconstituted CF_0F_1, treated with the procedures 4 and 5 with and without UV-irradiation are collected. These data show that about two 2-azido-[α-^{32}P]ADP are bound to the isolated CF_0F_1 with procedure 4 and one with procedure 5. During reconstitution, a small part (5–10%) of these nucleotides was released from the enzyme. Free ATP was not observed under any condition described. The data indicate that some of the endogenous bound ATP is converted to ADP and the latter is partially released during reconstitution. Correspondingly, the total nucleotide content (endogenous and 2-azido-[α-^{32}P]- AXP) decreased during reconstitution. Analysis of the derivatised peptides shows that with both procedures more than 95% of the label is nitreno-[α-^{32}P]ADP bound at a catalytic site (Tyr-362).

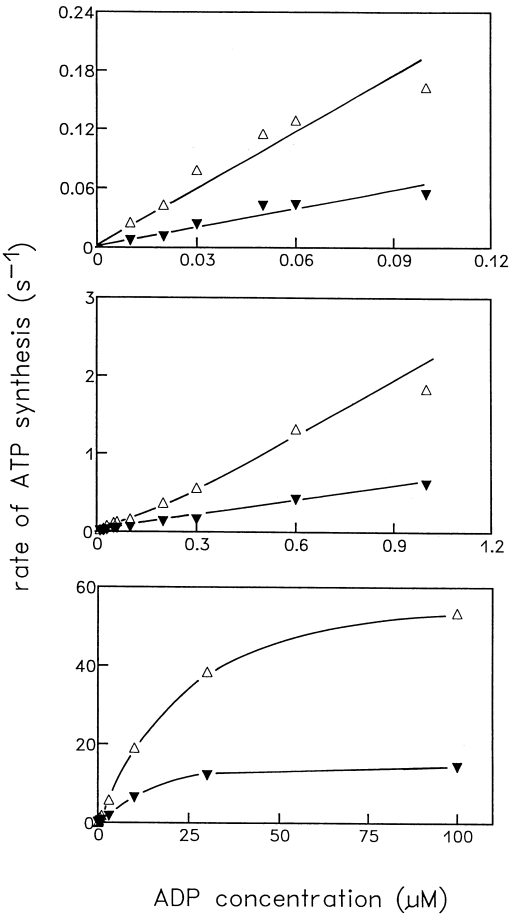

Fig. 7. ATP synthesis of CF_0F_1 with covalently bound and non-covalently bound 2-azido-[α-^{32}P]ADP at site 1 at different ADP concentrations. CF_0F_1 was treated according to procedure 5. After removal of free nucleotides, CF_0F_1 contained after reconstitution into liposomes 0.78 covalently bound 2-azido-[α-^{32}P]ADP (filled triangles) or 0.9 non-covalently bound 2-azido-[α-^{32}P]ADP (open triangles). The rate of ATP synthesis was measured after a $\Delta pH/\Delta\phi$ jump. Top panel: data under uni-site conditions (up to 100 nM ADP). Bottom panel: data under multi-site conditions (up to 100 μM ADP). Center panel shows the intermediate range.

3.7. Effect of covalent modification at site 1 on the kinetics of the enzyme

The enzyme was treated according to procedure 5 and covalently derivatised by 30 s of UV-irradiation. This resulted in covalent binding of 0.78 mol 2-nitreno-ADP per mol CF_0F_1, i.e. approximately 70% of the bound 2-azido-[α-^{32}P]ADP was covalently bound under these conditions. After reconstitution of the covalent derivatised and of the non-irradiated CF_0F_1

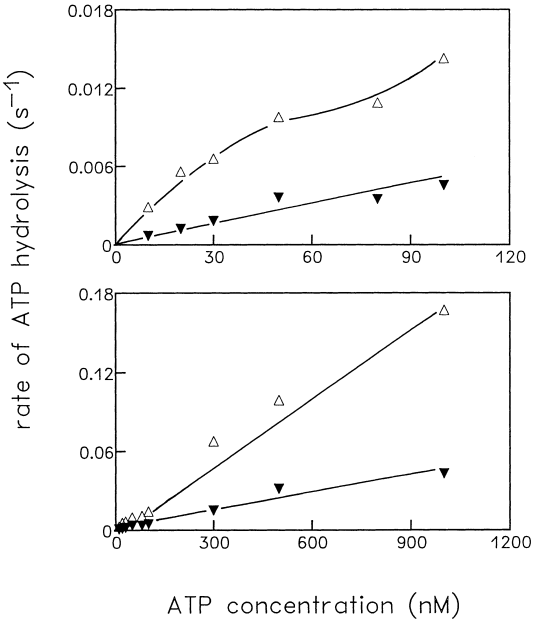

Fig. 8. ATP hydrolysis of CF_0F_1 with covalently bound and non-covalently bound 2-azido-[α-^{32}P]ADP at site 1 at different ATP concentrations. CF_0F_1 was treated according to procedure 5 and contained the same amount of 2-azido-[α-^{32}P]ADP as described in Fig. 6. The rate of ATP hydrolysis was measured after activation of the enzyme by a $\Delta pH/\Delta\phi$ jump from uni-site (upper panel) to multi-site (lower panel) conditions. Open triangles represent data with non-covalently bound 2-azido-[α-^{32}P]-ADP. Filled triangles represent data with covalently bound 2-azido-[α-^{32}P]ADP.

into liposomes, the rate of ATP synthesis was measured after a $\Delta pH/\Delta\phi$ jump.

Fig. 7 shows the rate of ATP synthesis for both enzymes as a function of the ADP concentration from uni-site conditions (top panel) to multi-site conditions (bottom panel). At high ADP concentrations covalent derivatisation leads to a decrease of the rate from 50 to 15 s^{-1}, i.e. to a decrease of about 70%. Under uni-site conditions (10 nM ADP) the rate decreases from 0.035 to 0.009 s^{-1}, i.e. also by 70%.

Fig. 8 shows the rate of ATP hydrolysis as a function of ATP concentration. At uni-site conditions (top panel), the rate decreases from 0.003 to 0.0005 s^{-1} after covalent derivatisation. Under multi-site conditions (1 mM ATP), the rate decreases from 17 to 5 s^{-1}, i.e. also by 70%. These data indicate that covalent derivatisation of the catalytic site 1 results in the same degree of inhibition of ATP synthesis and ATP hydrolysis, both under uni-site and under multi-site conditions. Considering the fact that about 70% of the bound 2-azido-[α-^{32}P-]ADP is covalently bound and the inhibition is 70%, the modification obviously, causes full inhibition of all activities of the modified enzyme molecules.

3.8. Release of non-covalently bound nucleotides by energisation

CF_0F_1 treated with procedure 5 or with procedure 4 contained after reconstitution in liposomes about 0.9 and 1.4 non-covalently bound 2-azido-[α-^{32}P]-ADP per enzyme. We investigated the release of the radioactive 2-azido-[α-^{32}P]ADP upon energisation of the membrane by $\Delta pH/\Delta\phi$ in the presence and in the absence of substrates. The data are shown in Table 3. Approximately 8% of the bound radioactive nucleotides are released in the absence of substrates and 20% in the presence of either ADP or ATP. This is surprising since during the time between energisation and separation of the enzyme from the released nucleotides (5 s), more than 500 ATP are newly synthesised per CF_0F_1 at an ADP concentra-

Table 3
Release of non-covalently bound 2-N_3-[α-^{32}P]AXP in $\Delta pH/\Delta\phi$ jump

Incubation conditions	Non-covalently bound 2-N_3-[α-^{32}]AXP	2-N_3-[α-^{32}P]AXP released in $\Delta pH/\Delta\phi$ jump without substrate	2-N_3-[α-^{32}P]AXP released in $\Delta pH/\Delta\phi$ jump	
			hydrolysis +1 mM ATP	synthesis +100 μM ADP
Procedure 4	1.4	nd	0.29	0.24
Procedure 5	0.95	0.08	0.18	0.16

CF_0F_1 (5 μM) was treated as described in procedure 4 or 5 and then reconstituted into liposomes. Energisation of the membrane leads to the release of the non-covalently bound 2-azido-[α-^{32}P]ADP. Free nucleotides were separated by rapid filtration from the liposomes 5 s after energisation. Experiments were carried out in the absence of substrates or in the presence of substrates, either 1 mM ATP or 100 μM ADP. Data are given in mol nucleotide per mol CF_0F_1; nd, not determined.

tion of 100 μM (see Fig. 7). In hydrolysis direction at 1 mM ATP, about 80 ATP per CF_0F_1 are hydrolysed within 5 s. If the radioactive nucleotide is located on a site involved in the catalytic reaction cycle, a complete loss of the label is expected within a few turnovers. Obviously, this was not the case; only 20–25% of the enzymes lost the label during turnover. We conclude from this result that only about 25% of the enzymes participate in the catalytic reaction cycle. Since all the rates given in this work are based on the amount of protein added during reconstitution, this finding implies either that only 20–25% of the CF_0F_1 complexes are structurally and functionally intact after isolation, or that only this percentage of the intact CF_0F_1 molecules are correctly inserted into the liposomes and are active in ATP synthesis and ATP hydrolysis.

3.9. Incubation of CF_0F_1 with 2-azido-[α-^{32}P]ATP

CF_0F_1 was incubated with 2-azido-[α-^{32}P]ATP under otherwise the same conditions as with 2-azido-[α-^{32}P]ADP. The results are shown in Fig. 9. During the first hour, about one mol 2-azido-nucleotide per mol CF_0F_1 is bound additionally, so that the total nucleotide content increases. This phase is followed by a release of almost all endogenous nucleotides and a binding of three mol 2-azido-nucleotides per mol CF_0F_1 after about 20 h. After UV-irradiation and tryptic digestion, the anion exchange chromatography shows -AMP-, -ADP- and -ATP-containing peptides. In the reversed phase chromatography the -AMP- and -ADP-labelled peptides elute at the position of the catalytic site tryptic peptide and sequence analysis indicates derivatisation of β-Tyr-362. The -ATP-labelled peptide elutes at a different position, and sequence analysis indicates that in this peptide β-Tyr-385 is derivatised. Since upon incubation with 2-azido-[α-^{32}P]ATP both catalytic and non-catalytic sites of the enzyme are modified, this CF_0F_1 was not used for kinetic analysis.

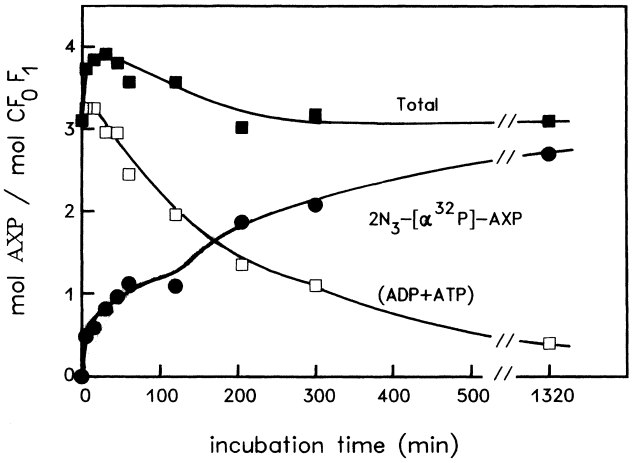

Fig. 9. Binding of 2-azido-[α-^{32}P]ATP to CF_0F_1. Isolated CF_0F_1 (5 μM) was incubated with 100 μM 2-azido-[α-^{32}P]ATP in the presence of 2 mM EDTA up to 22 h. After different incubation times, free nucleotides were removed by three consecutive passages through centrifugation columns. After different incubation times, the enzyme was denatured and nucleotide content was determined. Filled squares represent total nucleotide content, open squares endogenous ADP+ATP and filled circles 2-azido-[α-^{32}P]AXP.

4. Discussion

4.1. Occupation of the different nucleotide binding sites

In earlier work, the binding affinities, the exchangeabilities of nucleotides and Mg^{2+} requirement were used to characterise nucleotide binding sites [25,26,37]. As introduced by Xue et al. [14,15], we used the labelling of β-Tyr-362 by 2-azido-[α-^{32}P]AXP as an indication for binding at a catalytic site. With this approach, we found the following properties of the catalytic sites in isolated CF_0F_1: incubation with 2-azido-[α-^{32}P]ADP in the presence and absence of Mg^{2+} leads to selective labelling of β-Tyr-362. The nucleotide bound at the β-Tyr-362-containing peptide was predominantly ADP (>95%) and some AMP. Incubation with 2-azido-[α-^{32}P]ATP also leads mainly to labelling of β-Tyr-362; however, also some labelled β-Tyr-385 was found. Binding of 2-azido-[α-^{32}P]ATP occurred in the presence as well as in the absence of Mg^{2+}. The covalently bound nucleotide at β-Tyr-362 was also in this case predominantly ADP (>95%) and some AMP, while the covalently bound nucleotide at the non-catalytic site (β-Tyr-385) was ATP (>95%).

These data show that isolated CF_0F_1 binds ADP or 2-azido-ADP selectively at catalytic sites of the β-subunits. ATP and 2-azido-ATP bind to both catalytic and non-catalytic sites. When 2-azido-ATP

is bound to non-catalytic sites, it is not hydrolyzed even during the trypsin treatment of the CF_0F_1. When, however, 2-azido-ATP is bound to a catalytic site, it is hydrolyzed to 2-azido-ADP, so that only nitreno-ADP-modified β-Tyr-362 is found. In this work, we have used incubations with 2-azido-[α-^{32}P]ADP for selective labelling of catalytic sites. Based on these observations, we can interpret the distribution of the initially bound nucleotides on CF_0F_1. The CF_0F_1 used in this work contained one ADP and two ATP per enzyme molecule after isolation, and we conclude that ADP is bound at a catalytic site while the two ATP's are bound at non-catalytic sites.

The nucleotide binding sites have different properties. In order to distinguish the different catalytic sites, we call them sites 1, 2 and 3 in order of decreasing affinity for ADP, i.e. site 1 has the lowest dissociation constant for ADP, site 3 has the highest and site 2 has an intermediate dissociation constant [28]. Correspondingly, the three non-catalytic sites are called 4, 5 and 6 in order of decreasing affinity for ATP. This nomenclature was also used for description of the binding sites on MF_1 [38] and EF_1 [39]. Based on this classification, it is clear that the ADP at the catalytic site is localised on site 1 since, per definition, this is the site with the lowest dissociation constant. Similarly, the ATP on non-catalytic sites must be localised on sites 4 and 5, since these have the lowest and the intermediate dissociation constant. These considerations lead to the distribution of initially bound nucleotides depicted in Fig. 2, top.

When a sample of isolated CF_0F_1 with this nucleotide occupation pattern is incubated with 2-azido-[α-^{32}P]ADP, the ADP analogue binds to the catalytic site 2, i.e. the site with the intermediate dissociation constant. Also site 3 will bind the analogue, but at this site the nucleotide is not retained after column centrifugation. Prolonged incubation leads to the release of ATP from the enzyme (see Fig. 1). Since site 5 is the occupied non-catalytic site with the intermediate dissociation constant and site 6 is not occupied, the release of ATP occurs from site 5. Partly in parallel, but somewhat slower, binding of a second 2-azido-[α-^{32}P]ADP is observed, while one ADP is released. This slow exchange occurs at site 1, either direct or indirect. Removal of the free and loosely bound nucleotides at this incubation time (5 h, see Fig. 1) then leads to the occupation pattern shown in Fig. 2, centre: two 2-azido-[α-^{32}P]ADP are bound at the catalytic sites with the lowest (site 1) and the intermediate (site 2) dissociation constant. The result of procedure 4 is, therefore, occupation of the two catalytic sites with the lowest dissociation constants. Sequence analysis of the tryptic peptides after derivatisation indicates that only catalytic sites are occupied.

When an incubation with ADP is carried out with the enzyme preparation treated according to procedure 4, binding of ADP to site 3 occurs. Three catalytic binding sites are now occupied, the binding properties of site 2 are changed so that the bound 2-azido-[α-^{32}P]ADP is released and replaced by ADP (see Table 1). We then obtain the occupation pattern shown in Fig. 2, bottom. Sequence analysis of the tryptic peptides shows that only a catalytic site was derivatised. As result of procedure 5, we thus retain one 2-azido-[α-^{32}P]ADP at the site with the lowest dissociation constant (site 1), while an ADP is bound at the site with the intermediate dissociation constant (site 2). This conclusion is supported by the analysis of the inhibition of ATP synthesis and ATP hydrolysis after illumination. Covalent derivatisation of site 2 leads to inhibition of multi-site catalysis, whereas uni-site ATP synthesis and ATP hydrolysis are not impaired [28]. Covalent derivatisation of the enzyme treated according to procedure 5 leads to inhibition of uni-site and multi-site catalysis, i.e. a site with properties different from site 2 is modified. Since site 3 is not occupied after procedure 5, the 2-azido-[α-^{32}P]ADP must be located on site 1 and the ADP on site 2.

Site 1 is the site with the lowest dissociation constant and the question is whether 2-azido-[α-^{32}P]-ADP is bound directly to this site. The observed exchange at site 1 can be explained by assuming three interacting catalytic sites: when three catalytic sites are occupied by ADP or 2-azido-ADP in the absence of Mg^{2+}, a change in the binding affinities of all three sites may occur. In our case the ADP-containing site 1 (with lowest K_d) then changes to site 3 (with highest K_d). Correspondingly, the sites with the two bound 2-azido-[α-^{32}P]ADP's switch to site 1 (lowest K_d) and site 2 (intermediate K_d). Removal of nucleotides by three passages through

centrifugation columns then results in a loss of bound ADP and the enzyme contains 2 mol 2-azido-[α-^{32}P]ADP per mol CF_0F_1 on the new sites 1 and 2.

Since, however, the sequential adoption by each binding site of the three different conformations that are present in the enzyme, as proposed by the binding change mechanism, only occurs during the process of catalysis and the isolated CF_0F_1 is in an inactive state, another explanation is more likely. Various reports conclude that in CF_1 [40], *E. coli* F_1 [41] and TF_1 [42] site 1 can release and rebind a nucleotide without any interaction with the two other catalytic sites. Opening and closing of site 1 apparently does not require a conformational change at the other catalytic sites. The nucleotide on site 1, then, dissociates very slowly under the conditions of the incubation of procedure 4, so that after 5 h it is replaced in most enzyme molecules by a nucleotide from the medium. It should be mentioned that in the presence of Mg^{2+} the nucleotide at site 1 does not dissociate from its site and no exchange occurs. Interaction between sites 2 and 3, such that binding of an adenine nucleotide at site 3 results in a lower affinity of the nucleotide at site 2, followed by exchange of this nucleotide, has been quite firmly established [22] and in this way it was explained that a tightly bound nucleotide in MF_1 is exchanged with a K_d of 35–50 μM [38,43].

Our results show clearly that the inactive isolated CF_0F_1 contains three catalytic sites with different nucleotide binding affinities. It seems plausible to identify site 1 with the tight site, site 2 with the loose site and site 3 with the open site of the tri-site binding change mechanism [17,18,22,23]. However, one must keep in mind that the binding change mechanism describes the active enzyme during catalytic turnover, where the sequence of the affinity changes is determined by the direction of the chemical reaction. The procedures for selective occupation of the different conformations of catalytic sites described here and in [28] make use of the different ADP-binding affinities of the non-activated CF_0F_1. In this case, no catalytic reaction takes place and a possible effect of binding of a nucleotide at one site on the affinity of the nucleotide at another site does not give information on the interactions occurring during catalysis.

In addition to the exchange of nucleotides at the catalytic sites, we also observed a slow hydrolysis of ATP bound at the non-catalytic site 4. Therefore, in the scheme of Fig. 2, is site 4 occupied by ATP and ADP. The nucleotide distribution on non-catalytic sites and its effect on catalysis will be described in a forthcoming paper.

4.2. *Release of non-covalently bound 2-azido-[α-^{32}P]ADP during energisation*

Energisation of the liposome membrane by ΔpH/Δφ induced the release of 8% of the non-covalently bound 2-azido-[α-^{32}P]ADP from site 1, i.e. energisation generates an enzyme conformation which is characterised by a higher dissociation constant of nucleotides bound to site 1 as compared to the non-energised form. When energisation was carried out in the presence of substrates, approximately 20% of non-covalently bound 2-azido-[α-^{32}P]ADP were released. When all enzymes were involved in catalysis, a complete loss of the non-covalently bound label would be expected within a few turnovers. We must conclude from this result that only 20% of the enzymes are involved in the catalytic reaction. On the other hand, the stoichiometry and the kinetics of the binding of 2-azido-[α-^{32}P]ADP (Fig. 1) and the subsequent analysis of the covalently labelled peptides showed that a homogeneously labelled CF_0F_1 preparation was obtained. Therefore, we assume that the heterogeneity of the response of CF_0F_1 to membrane energisation results from the reconstitution procedure, i.e. only 20–25% of the enzymes added for reconstitution are correctly inserted into the membrane, and only these are involved in catalysis. The rest is not reconstituted or not functionally inserted into the membrane. All enzyme activities (turnover, rate) in this work are based on the amount of enzyme added to the reconstitution medium, assuming that all the enzymes are active in catalysis. If only 20% of the added enzymes are activated, all the rates given in this work have to be multiplied by a factor of 5 in order to obtain the rates based on the amount of correctly reconstituted enzymes. The rate of ATP synthesis per enzyme (added to the reconstitution medium) was about 90 s^{-1} and, therefore, the rate per active enzyme is expected to be 450 s^{-1}. Such rates have been measured when thylakoid membranes are energised by ΔpH/Δφ [36].

4.3. Covalent labelling and inhibition of catalysis

UV-irradiation of CF_0F_1 for up to 30 s before reconstitution had no detectable effect on the rate of ATP synthesis, irradiation for 45 s caused a decrease of about 10%. Non-covalent binding of 2-azido-[α-^{32}P]ADP to site 1 or to sites 1 and 2, before reconstitution, did not affect the rate of ATP synthesis. Covalent binding of 2-azido-[α-^{32}P]ADP before reconstitution inhibited ATP synthesis proportional to the degree of labelling. When only site 1 is labelled, the extrapolation indicates complete inhibition when one nucleotide is covalently bound (Fig. 3). For sites 1 and 2, extrapolation reveals that inhibition is complete when two nucleotides per enzyme are covalently bound (Fig. 3). This result is surprising since bound nitreno-radical, formed upon illumination, should have a probability to react with β-Tyr-362 or with water that is independent of what happens at other sites. The efficiency of the formation of a covalent bond appears to be about 70% at site 1 (this work) and site 2 [28]. When two sites are occupied, this efficiency remains the same, since 1.4 mol become covalently linked when sites 1 and 2 are occupied (see Table 1). Since one covalently bound nucleotide is sufficient to block completely multi-site catalysis and some enzymes contain two bound nucleotides, a non-linear relation between activity and the amount of covalently bound nitreno-ADP is expected. This is obviously not observed. The most probable explanation seems to be that the covalent derivatisation at one site induces a slight conformational change at the second site such that, upon formation of the nitreno-radical, in almost all cases, a covalent bond with the tyrosine is formed. Correspondingly, in the enzyme molecules in which at the first site the nitreno-ADP has not reacted with Tyr-362, but with water, also the nitreno-ADP formed at the second site reacts almost exclusively with water.

For comparison, Garin et al. [44] have investigated the inhibition of catalysis by binding of 2- and 8-azido-ADP-fluoroaluminate complexes. In this case, two catalytic sites become occupied by an azido-ADP-fluoroaluminate complex, supposedly independent of each other, but the inhibition curve is completely linear, extrapolating to full inhibition when two sites are occupied. The reason for this effect is not clear, but it might indicate that there is a highly (positive) cooperative binding of the inhibitors.

The relation between inhibition and covalent derivatisation with 2-azido-nucleotides has been extensively studied with the F_1-parts from different sources [11,24,31,45–48]. In most cases, one covalently bound nucleotide was sufficient to inhibit ATP hydrolysis, however, in some cases two nucleotides were required for complete inhibition and not always simple correlations have been found (for review of earlier work see [22,23]). Interestingly, in *E. coli* F_1 inhibition of multi-site and uni-site ATP hydrolysis was observed after reaction with 1 mol adenosine triphosphopyridoxal per mol F_1 [49]. Recently, the mitochondrial H^+-ATPase was labelled with 2-azido-nucleotides in submitochondrial particles and a complete inhibition of ATP synthesis and ATP hydrolysis (under multi-site conditions) was found when one catalytic site was derivatised [50].

We have used CF_0F_1 for our investigations because this enzyme has several advantages. First, the effect of covalent derivatisation on the complete reaction cycle can be studied. Second, the isolated CF_0F_1 is catalytically inactive. Therefore, enzyme species with well-defined nucleotide occupation patterns can be prepared, and this distribution does not change significantly during the handling of the enzyme. Third, the rate of ATP synthesis is used for assaying the catalytic activity, i.e. we measure only enzymes which are functionally reconstituted into the membrane. For interpretation of the data we have to assume that the reconstitution efficiency is the same for derivatised and non-derivatised CF_0F_1. Fourth, both uni-site and multi-site catalysis can easily be measured.

Our data indicate that covalent derivatisation of catalytic site 1 inhibits completely both multi-site and uni-site catalysis catalysis (ATP synthesis as well as ATP hydrolysis). Derivatisation of either catalytic site 2 or site 3 leads to inhibition of multi-site catalysis, whereas uni-site catalysis is not impaired [28]. Obviously, labelling of site 2 or site 3 abolishes the cooperativity between catalytic sites, which is required for multi-site catalysis, but still allows uni-site catalysis at one of the non-derivatised sites. The fact that labelling of site 1 abolishes both multi- and uni-site catalysis might imply that uni-site catalysis can

proceed only at site 1, but it seems more plausible that the covalently bound ADP makes an activation of CF_0F_1 by membrane energisation impossible.

CF_0F_1 bound at thylakoid membranes contains a tightly bound ADP which is released upon membrane energisation [51–54]. CF_0F_1 is in an inactive form in deenergised membranes and energisation leads to a conformational change of the enzyme. This results in the release of ADP and, thereby, the enzyme is brought into the metastable active state. The fraction of released ADP gives the fraction of active enzymes. Correspondingly, when the membrane is deenergised, rebinding of ADP inactivates the enzyme and this process can be measured by its effect on the ATP hydrolysis activity [21,55,56]. Derivatisation of site 1 prevents the release of ADP and, thereby, the activation of the enzyme.

The effect of modification of site 1 differs significantly from that of the two other sites. Obviously, one β-site differs from the two others and this might imply that site 1 is located on the β-subunit interacting with the δ, I, II-subunits (the stator, [57,58]) and/or with the γ-ε-subunits. Such a structural asymmetry might be the reason for the differing binding affinities of the β-sites in the inactive enzyme, but in the active state, binding affinities might change during catalysis in such a way that all three sites are equally participating in multi-site catalysis [17,18,22]. The results reported here are therefore compatible with a two-site mechanism (in this case, the asymmetry found in the inactive enzyme remains during catalysis) as well as with a three-site mechanism (in this case, activation leads to a symmetrisation of the catalytic sites). Nevertheless, we would like to point out that earlier data indicate heterogeneity between site 1 and sites 2 and 3 also during catalysis [20,21] and a recent study shows that the catalysis that is characterised by a low rate and a low K_m (properties of uni-site catalysis at site 1) proceeds fully independent of the catalysis that is characterised by a high rate and a high K_m [40].

Acknowledgements

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